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PERIPHERAL SECRETIN-INDUCED FOS EXPRESSION IN THE RAT BRAIN IS LARGELY VAGAL DEPENDENT

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Running title: Vagotomy blocked secretin-induced brain Fos expression

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Abbreviations: AP, area postrema; Arc, arcuate nucleus; Bar, Barrington's nucleus; CeA, central nucleus of the amygdala; DMV, dorsal motor nucleus of the vagus; DVC, dorsal vagal complex; iv, intravenous; LC, locus coeruleus; NTS, nucleus tractus solitarii; PBel, external lateral subnucleus of the parabrachial nucleus; Real-time-PCR, quantitative real-time polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; SecR, secretin receptor; TH, tyrosine hydroxylase.

Abstract-- Intravenous (iv) injection of secretin activates neurons in brain areas controlling autonomic function and emotion. Peripheral administration of secretin inhibits gastric functions through a central mechanism that is mediated by vagal dependant pathways. We investigated whether the vagus nerve is involved in intraperitoneal (ip) injection of secretin-induced brain neuronal activation in conscious rats as monitored by Fos immunohistochemistry. Secretin (40 or 100 μg/kg, ip, -90 min) induced a linear dose-related increase in the number of Fos positive neurons in the central nucleus of the amygdala (CeA), and a plateau Fos response in the area postrema (AP), nucleus tractus solitarii (NTS), locus coeruleus (LC), Barrington's nucleus (Bar), external lateral subnucleus of parabrachial nucleus (PBel) and arcuate nucleus (Arc), and at the highest dose, in the dorsal motor nucleus of the vagus (DMV) compared with ip injection of vehicle. Double immunohistochemistry in the NTS showed that a substantial number of neurons activated by secretin (40 μg/kg, ip) are tyrosine hydroxylase positive. Subdiaphragmatic vagotomy (-7 days) abolished Fos expression-induced by ip secretin (40 μg/kg) in the NTS, DMV, LC, Bar, PBel and CeA, while a significant rise in the AP was maintained. In contrast, capsaicin (-10 days) did not influence the Fos induction in the above nuclei. Quantitative real-time polymerase chain reaction (PCR) and reverse transcription PCR showed that secretin receptor mRNA is expressed in the nodose ganglia and levels were higher in the right compared to the left ganglion. These results indicate that peripheral secretin activates catecholaminergic NTS neurons as well as neurons in pontine and limbic nuclei regulating autonomic functions and emotion through vagal dependent capsaicin resistant pathways. Secretin injected ip may signal to the brain by interacting with secretin receptors on vagal afferent as well as on AP neurons outside the blood brain barrier.

Key words: capsaicin; secretin receptor; nodose ganglia; vagus; nucleus tractus solitarii; central nucleus of the amygdala.

Secretin is a 27-amino acid neuropeptide, member of the secretin/glucagon/vasoactive intestinal polypeptide super-family (Chey et al., 2002). Secretin is released into the circulation from the endocrine S cells in the proximal small intestine when the pH in the duodenal lumen falls below 4.5 (Chey et al., 2002). Besides its regulatory actions on digestive functions, secretin is also thought to act as a central neuromodulator (Ng et al., 2002). In 1998, a published case series described the beneficial therapeutic effect of secretin in 3 children with autism (Horvath et al., 1998; Volkmar, 1999), a neuronal developmental disorder with poorly understood etiology (Korvatska et al., 2002; Sweeten et al., 2002). However, other studies failed to reproduce this finding (Chez et al., 2000; Sandler et al., 1999). More recently, another case series documented improvements in communication and social functioning after secretin treatment in schizophrenic patients (Alamy et al., 2004; Sheitman et al., 2004). One of the characteristics of autism and schizophrenia is the over-reaction to external stimuli, sometimes resulting in anxiety or even panic (Kaminska et al., 2002). Since autism and schizophrenia are believed to share neurodevelopmental abnormalities, it can be entertained that secretin may influence similar if not identical brain circuits affected in these neurological disorders (Alamy et al., 2004). The limbic system, in particular the amygdala, has been implicated in the coding of emotional and species-specific social behavior (Adolphs et al., 2002; Baxter et al., 2002; Brothers et al., 1990; Prather et al., 2001). It has been suggested that dysfunction of amygdala may be involved in the abnormal fears and increased anxiety in autistic children and defects in sensory information processing in schizophrenia patients (Amaral et al., 2003; Bakshi et al., 1998). Recent experimental studies by Goulet et al. (2003) and Welch et al. (2003) showed that intravenous (iv) or intracerebroventricular injection of secretin in rats induced the most robust neural activation in the central nucleus of the amygdala (CeA) as shown by Fos expression, a well-established

marker of neuronal pathways tracing (Dragunow et al., 1989). Other specific brain nuclei displaying significant increase in the number of Fos positive neurons after iv injection of secretin compared with iv vehicle included the area postrema (AP), bed nucleus of the stria terminalis and supraoptic nucleus, and to a smaller extent the dorsal motor nucleus of the vagus (DMV) and medial region of the nucleus tractus solitarii (NTS) (Goulet et al., 2003).

Very little is known regarding the signal transduction mechanisms through which peripheral secretin influences the activity of selective brain nuclei. Previous studies identified the presence of secretin binding sites in specific brain areas, most notably in the NTS (Nozaki et al., 2002). Other studies demonstrated that peripheral secretin can penetrate into the brain in an intact form (Banks et al., 2002). Our previous studies on gut-brain interactions showed that peripheral administration of gut peptides such as cholecystokinin (CCK), bombesin, peptide YY, corticotropin releasing factor, urocortin and ghrelin induces selective patterns of Fos expression in brain nuclei that are either outside of the blood brain barrier (BBB), such as the AP in the medulla and the arcuate nucleus (Arc) in the hypothalamus, or key areas receiving peripheral afferent signaling, such as the NTS (Bonaz et al., 1993a; Bonaz et al., 1993b; Wang et al., 1998; Wang et al., 2000; Wang et al., 2002). Other brain sites, such as the CeA, and external lateral parabrachial nucleus (PBel), that are involved in the autonomic and emotional regulations and closely linked to the AP, NTS and Arc, were activated by peripheral administrations of these peptides as well (Bonaz et al., 1993a; Bonaz et al., 1993b; Wang et al., 1998; Wang et al., 2000; Wang et al., 2002). The brain responses to some peripherally administered gut peptides, such as CCK and ghrelin, are mediated through vagal afferent pathways (Date et al., 2002; Li et al., 1995; Monnikes et al., 1997). Subdiaphragmatic vagotomy was shown to completely abolished intraperitoneal (ip) CCK-induced Fos expression (Li et al., 1995). Likewise, the gastric

inhibitory effects of secretin administered peripherally at low doses are mediated through a central mechanism where capsaicin sensitive vagal afferents serve as the pathway to transfer peripheral secretin signals to the brain (Li et al., 1998; Lu et al., 1995; Raybould et al., 1993).

In the present study, we investigated in conscious rats the extent to which the vagus nerve mediates brain neuronal activation induced by secretin injected peripherally (ip) at a dose that reproduces peak blood levels of the peptide during administration to autistic patients (Goulet et al., 2003). First, we monitored Fos expression induced by ip secretin in brain medullary areas receiving vagal input and limbic and pontine areas involved in anxiety and fear. We also examined whether ip secretin activates catecholaminergic neurons in the A2 (in the NTS) and A6 (LC) regions since these cell groups are known to provide catecholaminergic input to the CeA (Myers et al., 2002; Zardetto-Smith et al., 1990). Secondly, we determined the importance of vagal integrity and capsaicin sensitive fibers in mediating ip secretin-induced Fos expression in specific brain nuclei. Finally, quantitative real-time polymerase chain reaction (real-time-PCR) and reverse transcription-PCR (RT-PCR) were used to detect and measure the expression of secretin receptors in the nodose ganglia, where vagal afferent neurons are localized (Berthoud et al., 1995).

EXPERIMENTAL PROCEDURES

Animals

Male Sprague-Dawley rats (Harlan Laboratory, San Diego, CA) weighing 280-355 g were housed under controlled conditions (21-23°C, light on from 6.00 a.m. to 6.00 p.m.). Animals had free access to standard rat chow (Prolab RMH 2500, PMY Nutrition International, Brentwood, MD) and tap water. The experiments were carried out in accordance with the National Institute

of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). Animal experimental protocols were approved by the UCLA Office for the Protection of Research Subjects and VA GLAHS Animal Committee.

Compounds and treatments

Synthetic human secretin (Repligen Corp., Waltham, MA) was dissolved in a vehicle containing 10 mM sodium citrate, 2.0 mg/ml D-mannitol and 0.004% Tween 80 just before being injected ip at 40 or 100 μg/kg. Capsaicin (Sigma Chemical, St. Louis, MO) was dissolved at the concentration of 12.5 mg/ml in a vehicle containing 10% Tween 80, 10% ethanol and 80% normal saline. Capsaicin or vehicle was injected subcutaneously (sc) at a total dose of 125 mg/kg, which was divided into three injections, 25 mg/kg in the morning (under short anesthesia with isoflurane at 5% vapor concentration in oxygen) and 50 mg/kg in the afternoon of the 1st day followed by 50 mg/kg on the 2nd day in conscious rats. Effectiveness of capsaicin treatment was tested by the eye wiping test in which eye wiping in response to a drop of 0.1% ammonium hydroxide instilled into one eye of rats pretreated with capsaicin 10 days before was monitored for 2-3 min. The disappearance of the corneal chemosensory reflex indicates the effectiveness of the capsaicin treatment and only those rats showing the disappearance of the reflex were used.

Subdiaphragmatic vagotomy

Subdiaphragmatic vagotomy was performed in 24-h fasted rats under anesthesia induced by a 3:1 volume mixture of ketamine (75 mg/kg; Fort Dodge laboratories, Fort Dodge, IA) and xylazine (5 mg/kg; Mobay Corporation, Shawnee, KS). The animal underwent a midline abdominal incision and the liver was gently retracted from the stomach using a cotton-tipped

applicator. The lower esophagus was then exposed by gentle traction of the stomach. Approximately 0.5 cm of both gastric vagal branches were resected under a stereomicroscope by seromyotomy using forceps. Sham-operated animals underwent the same procedure without resecting the vagal branches. The midline incision was then closed in layers and the animals kept for recovery on the heating pad. Sham and vagotomized rats was then housed individually in Plexiglas cages and kept under controlled environmental conditions mentioned above. A grid was disposed on the floor of each cage to avoid contact of the surgical wound with bedding material. After sham or vagotomy, animals were fed with a liquid diet (Ensure, Abbot Laboratories, Columbus, OH) to prevent the delay of gastric emptying of solid food, which is occurred in vagotomized rats. The body weights of vagotomized rats were not different from those of the sham operated animals (344.5 ± 4.6 vs 351 ± 2.4 g) at 7 days after the surgery.

Experiment protocols

All experiments were performed in conscious non-fasted rats between 10 am and 2 pm to avoid any circadian influence on the parameters monitored.

Dose-related effects of ip secretin on Fos expression in the brain. Rats were injected ip with either vehicle (10 mM sodium citrate, 2.0 mg/ml D-mannitol and 0.004% Tween 80) or secretin (40 or 100 μg/kg) and 90 min later, were transcardially perfused with fixative. The brain was collected for Fos immunohistochemistry and double labeled forFos and TH in the NTS.

Effect of subdiaphragmatic vagotomy on ip secretin-induced Fos expression in the brain. Rats underwent sham operation or subdiaphragmatic vagotomy and 7 days later were injected ip with either vehicle (10 mM sodium citrate, 2.0 mg/ml D-mannitol and 0.004% Tween 80) or secretin

(40 μg/kg). At 90 min after the ip injection, rats were transcardially perfused and the brain collected for Fos immunohistochemistry.

Effect of capsaicin pretreatment on ip secretin-induced Fos expression in the brain. Conscious non-fasted rats received sc pretreatment with either vehicle (10% Tween 80, 10% ethanol and 80% of normal saline) or capsaicin (125 mg/kg). Then 10 days later, rats received either ip vehicle or secretin (40 μg/kg) and were transcardially perfused at 90 min after the injection. The brain was collected for Fos immunohistochemistry.

Transcardial perfusion and brain tissue preparation

Rats were deeply anesthetized with pentobarbital (70 mg/kg, ip, Abbott Laboratories, North Chicago, IL). The thoracic cavity was opened and the rat was transcardially perfused with 50 ml of isotonic saline (pH 7.4) followed by 500 ml of fixative containing 4% paraformaldehyde and 14% saturated picric acid in 0.1 M sodium phosphate buffer (PB, pH 7.4). The brain was collected after the perfusion. Brains were post-fixed for 4 h at 4°C in the same fixative, and subsequently cryoprotected overnight in 20% sucrose in 0.1 M PB. Coronal frozen sections (30 μm) were cryostat cut (Microtome, IEC, MA) from the interaural levels of –4.24 to 7.30 mm according to the atlas of Paxinos and Watson (Paxinos et al., 1997). Brain sections were collected in 0.01 M phosphate buffer saline (PBS).

Immunohistochemistry

Immunohistochemistry for Fos. Fos immunostaining was performed as previously described (Bonaz et al., 1993b; Wang et al., 1998; Yuan et al., 2002). Briefly, brain sections were rinsed in PBS and incubated for 30 min in 3% normal goat serum followed by a 24 h incubation at 4°C

with a polyclonal rabbit anti-Fos serum (Fos Ab-5, Oncogene Research Products, Cambridge, MA) diluted at 1:10,000 in PBS containing 0. 3% Triton X-100 (PBS-T, pH 7.4), then rinsed in PBS and incubated for 2 h at room temperature with biotinylated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA; 1:1000), followed by 1 h incubation with avidin-biotin-horseradish peroxidase complex (ABC) at 1:400 . The coloration of immunostaining was processed byperoxidase -catalyzed reaction of substrates with presenting 3,3'-tetrachloride diaminobenzidine (DAB) and H_2O_2. Fos immunoreactivity (IR) was detected as a dark brown nuclear staining. Immunohistochemical controls were routinely performed following the same procedure except that the primary antibody was replaced by PBS-T.

Double immunostaining of Fos and tyrosine hydroxylase (TH). After being processed for Fos immunohistochemistry as described above, except that DAB was enhanced with nickel ammonia sulphate, brainstem sections containing NTS were rinsed in 0.01 M PBS for 3 h at room temperature and then incubated overnight at 4°C with a mouse monoclonal antibody raised against TH (Boehringer Mannheim, 1 017 381; 1:2000) followed by biotinylated goat anti-mouse IgG (Jackson ImmunoResearch; 1:1000). Sections then were incubated with ABC for 1 h with DAB as chromogen. Fos-IR was detected as a dark blue reaction product in the nuclei and TH-IR appeared as a brown reaction product in the cytoplasm.

Quantitative analysis and statistics. The numbers of Fos-IR cells were counted under microscopy. In each rat, the mean number of Fos positive cells per brain sections covering each brain nucleus was calculated from a number of sections at specific interaural levels according to the atlas of Paxinos and Watson (Paxinos et al., 1997): DMV/NTS/AP, 20 sections (–4.24 to –5.08 mm, bilaterally); LC and Barrington's nucleus (Bar), 10 sections (-0.50 to –1.04 mm, unilaterally); PBel, 7 sections (0.00 to 0.25 mm, unilaterally); Arc, 20 sections (5.70-6.70 mm,

bilaterally) and CeA, 15 sections (6.20 to 7.00 mm, unilaterally). The mean number of Fos positive cells per section from each animal was used to calculate the group mean. Data are expressed as mean ± SEM of the number of Fos-positive cells per section in a particular brain nucleus. Comparisons among groups' mean values were performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test and between two groups using Student's *t*-test. $P < 0.05$ was considered statistically significant. Photomicrographs of brain sections representative of treatment effects in different groups were acquired under identical conditions with C·Imaging System (Compix, Inc.) connected to Zeiss Axioskop2 microscope and Hamamatsu digit camera C4742-95 (Hamamatsu Photonics K.K.).

Real-time-PCR and RT-PCR of secretin receptor mRNA expression in the nodose ganglia

The rat cerebellum and nodose ganglia were dissected from 6 naïve rats that were euthanized by decapitation. Tissues were frozen on dry ice and stored at 80°C until RNA isolation. Total RNA was isolated using RNeasy minicolumns (Qiagen, Valencia, CA), 1/5 of each nodose ganglion RNA was reversely transcribed with Superscript II (Invitrogen, Carlsbad, CA). Nodose ganglion cDNA from each RT reaction was used for PCR of secretin receptor and control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (amplicon size= 523 bp) or acetic ribosomal protein (ARP, amplicon size= 402 bp). After an initial denaturation step at 94°C for 2 min, the PCR conditions were as follows: 1 min at 94°C, 45 sec at 60°C and 45 sec at 72°C for 30 cycles. The first pair of secretin receptor primers was designed to amplify the entire N-terminal extracellular domain (amplicon size= 492 bp). Secretin receptor-1 (SecR-1) (sense) 5'-GGGCCAGAACGCAAAGGGGAGCAAC; SecR-2 (anti-sense) 5'-GAACTGTAGCCTACAGTGTACATGAC. Additionally, a second pair of primers was used to

generate PCR product encompassing region between TM-3 and TM-6 (amplicon size = 344 bp). SecR-3 (sense) 5'- GGCTGCTGGTGGAGGGCCTCT; SecR-4 (anti-sense) 5'-CAGGAGGGTGGACTTGGCCAG. Primers for control genes: GAPDH (sense) 5'-GTGAACCACGAGAAATATGACAACTCC; GAPDH (anti-sense) 5'-TCACAAAGTTGTCATTGAGAGCAATGC. ARP (sense) 5'-GTTGAACATCTCCCCCTTCTC; ARP (anti-sense) 5'-ATGTCCTCATCGGATTCCTCC. PCR products were resolved by agarose gel electrophoresis in the presence of ethidium bromide followed by visualizing under UV illumination. The fluorescent gel image was then captured by a Kodak imaging system and analyzed by image analysis software (Kodak 1D Image Analysis, Rochester, NY).

For TaqMan analysis, cDNA synthesis was carried out using random primers and Superscript II (Invitrogen). PCR primers and TaqMan probes were selected using the Primer Express 1.0 Software program (Applied Biosystems, Foster City, CA). Primers and probe sequences are as follows: secretin receptor (amplicon size = 73 bp); forward primer 5'-GGGTTCTCCAGCCATTTTTG-3'; reverse primer 5'-GTCCCAGCACCAGTATTTTCTAGA-3'; TaqMan probe 6FAM5'-TGCTTTGTGGGCTATCACCAGGCAC-3'TAMRA and GAPDH (amplicon size = 111bp): forward primer 6FAM5'-GGTCATCAACGGGAAACCC-3'TAMRA; reverse primer 6FAM5'-GTGGTGAAGACGCCAGTAGACTC-3'TAMRA; TaqMan probe 6FAM5'-CCATCTTCCAGGAGCGAGATCCCG-3'TAMRA. Nucleotides that cross exon boundaries are underlined. PCR was performed using the TaqMan Universal PCR Master Mix, 400 nM of both primers, 80nM of TaqMan probe and 5-10% of reverse transcribed cDNA on the ABI PRISM 7700 Sequence Detection System according to the manufacturer (Applied Biosystems, Foster City, CA). The amplicons were sequenced to confirm analytical specificity.

For each sample, data was normalized to GAPDH and this value adjusted so that left nodose ganglion had a mean relative mRNA level of 1. Data are presented as mean ± SEM (Fig. 5C,5D). Statistical analysis of the difference in mRNA levels was performed by two-tailed student's t-test.

RESULTS

Intraperitoneal secretin induced Fos expression in selective brain nuclei

In conscious non-fasted rats, the number of Fos positive cells in selective brain nuclei (AP, NTS, DVM, LC, Bar, PBel, Arc, and CeA) monitored at 90 min after ip injection of vehicle was low (1-7 cells per section, Fig. 1). Secretin injected ip at 40 or 100 μg/kg induced a linear dose-related significant increase in the number of Fos positive cells in the CeA (cells/section: 31.8 ± 9.5 and 64.4 ± 10.4 respectively vs vehicle 6.8 ± 1.1, $P<0.05$), whereas the lateral, basolateral and medial amygdala regions showed no increase of Fos-positive neurons (data not shown). Fos-containing cells increased significantly after ip injection of secretin (40 μg/kg) from vehicle values of 1.6 ± 1.0 to 10.4 ± 2.2 in the AP, 2.5 ± 0.8 to 21.8 ± 7.3 in the NTS, 1.6 ± 0.5 to 8.0 ± 1.7 in the LC and 0.6 ± 0.3 to 24.1 ± 6.7 in the PBel. A similar increase of Fos expression in these nuclei was observed when secretin was injected at 100 μg/kg (Fig. 1). Secretin (40 or 100 μg/kg, ip) also induced weak Fos increases in the DMV (from vehicle group of 0.9 ± 0.4 to 1.4 ± 0.8 and 4.2 ± 1.3 respectively), Arc (from 6.6 ± 2.7 to 12.9 ± 3.9 and 16.3 ± 3.3) and Bar (from 2.9 ± 0.5 to 7.2 ± 1.7 and 8.2 ± 2.3), however, except the DMV response to secretin at 100 μg/kg, these changes did not reach statistical significance (Fig. 1).

Double immunostaining with Fos and TH in NTS neurons showed that a substantial number of TH positive neurons were Fos positive after ip secretin (40 μg/kg) (Fig. 2). The double labeled neurons were mainly localized in the medial, dorsomedial and commissural subnucleus of the NTS.

Subdiaphragmatic vagotomy blunts brain Fos expression induced by ip secretin

In sham operated rats fed a liquid diet for 7 days, Fos expression at 90 min after ip injection of vehicle was low (1-5 Fos positive cells) in the AP, DMV, LC, Bar, and CeA, while the number ranged between 9-12 cells in the NTS, Arc and PBel (Fig. 3). The higher basal levels of Fos expression in these nuclei may reflect sensory inputs triggered by the liquid diet in these nuclei that receive gastric input. Secretin (40 μg/kg, ip) significantly increased Fos expression in the AP (16.6 ± 3.0 vs 1.5 ± 0.5), NTS (32.9 ± 6.6 vs 9.3 ± 3.7), Bar (9.3 ± 1.7 vs 3.3 ± 1.9) and CeA (86.8 ± 17.5 vs 5.1 ± 2.5) compared with ip vehicle in sham operated group (Fig. 3). Subdiaphragmatic vagotomy performed 7 days before the experiment did not significantly influence the Fos expression after ip vehicle injection compared with sham operated group receiving vehicle ip injection. However, vagotomy abolished ip secretin-induced Fos expression in the DMV (Fos positive cells: 0.6 ± 0.3 in vagotomy + secretin vs 1.8 ± 0.4 in sham operation + secretin), LC (4.7 ± 1.5 vs 18.4 ± 5.1), Bar (1.8 ± 0.8 vs 9.3 ± 1.7), PBel (6.0 ± 1.4 vs 26.0 ± 9.9) and CeA (13.9 ± 7.8 vs 86.8 ± 17.5) (Figs. 3,4). The secretin-induced significant increase in Fos positive cells in the NTS was also abolished by subdiaphragmatic vagotomy (16.4 ± 3.7 vs 32.9 ± 6.6 in sham + secretin) (Fig.3). The Fos expression in the AP was still significantly increased after ip secretin in vagotomized rats compared with vagotomy + vehicle ip group (9.6

± 2.9 vs 1.6 ± 0.4) though the number of Fos positive neurons was reduced compared with sham-operated group injected ip with secretin (16.6 ± 3.0).

Capsaicin pretreatment did not influence ip secretin–induced Fos expression in the observed brain nuclei

In vehicle-pretreated rats, the number of Fos positive cells induced by ip secretin (40 μg/kg) in the observed nuclei was similar to that induced in the non-pretreated rats (Fig. 1 and Table 1). Capsaicin pretreatment did not alter Fos expression induced by ip secretin in the AP, NTS, DMV, LC, Bar, PBel and CeA compared with vehicle pretreated and secretin injected group (Table 1).

Secretin receptor mRNA are detected in nodose ganglia

The rat cerebellum is known to express secretin receptor mRNA (Yung et al., 2001). In real-time-PCR experiments**,** cerebellum tissue was thus used as a positive control (Fig. 5B). Secretin receptor mRNA was detected in both the left and right nodose ganglions by real-time-PCR and RT-PCR (Fig. 5A,5B). A set of primer located within the N-terminal extracellular domain was used for real-time-PCR whereas another one spanning transmembrane domains 3 and 6 was used for RT-PCR (Fig. 5A, 5B). Densitometic assessment confirmed that expression of secretin receptors in the right nodose ganglion was significantly higher than in the left by 40% (Fig. 5C). The ratio of secretin receptor mRNA in the right ($SecR_R$) to that in the left ($SecR_R/SecR_L$) in individual animals ranges from 1.21 to 1.68 (mean ± SE: 1.40 ± 0.07, n=6) (Fig. 5C). Real-time quantitative PCR results showed a 3-fold higher level of secretin receptor mRNA expression in the right compared to left nodose ganglion (Fig. 5D).

DISCUSSION

Secretin injected ip at 40 μg/kg activates neurons in selective brain nuclei as shown by the significant increase in the number of Fos positive cells in the AP, subnuclei of the NTS, LC, PBel and CeA at 90 min after injection compared with vehicle in freely moving, non-fasted rats. There was also a clear trend towards a rise in the number of Fos positive cells in the Bar and Arc nuclei. The increase of Fos expression is not related to the non-specific effect of ip injection since the number of Fos positive cells waslow in these nuclei after ip vehicle administration and increased dose-dependently in the CeA after ip secretin at 40 and 100 μg/kg. Secretin at 100 μg/kg, in addition to induce a similar magnitude of Fos expression in the NTS, AP, LC and PBel, activated a small population of DMV neurons and induced a robust response in the CeA. These observations extend our previous findings showing that secretin injected intravenously at 4 to 40 μg/kg induced a lineal dose-related Fos expression in the CeA with an intense labeling at the highest dose (Goulet et al., 2003). Secretin injected iv at 40 μg/kg increases significantly the number of Fos positive cells in the AP, bed nucleus of the stria terminalis, supraoptic nucleus and the DMV with a trend of increase in the NTS (Goulet et al., 2003). Collectively these data established that peripheral secretin injected either intraperitoneally or intravenously elicits a robust Fos expression in the CeA along with a pattern of Fos induction in selective brain medullary (AP, NTS and DMV) and pontine (LC, Bar and PBel) nuclei.

The pathways by which ip administered secretin signals the brain to activate these specific nuclei have not been conclusively identified. Recent studies in mice indicate that secretin injected iv enters the brain at a modest rate by a saturable transporter at the choroid plexus as

well as by transmembrane diffusion (Banks et al., 2002). However, it is important to note that if 0.12% of the secretin injected iv enters the brain (Banks et al., 2002) this would represent about 0.012 μg for a 40 μg/kg iv dose. Behavioral effects for secretin have been reported with as little as 0.001 μg infused icv (Babarczy et al., 1995). Therefore the amount of secretin reaching the brain after peripheral injection may not be sufficient to induce detectable Fos expression but may still be of biological relevance.

Secretin might act outside of the BBB at the level of circumventricular organs. In the brain medulla, the BBB is absent in the AP and weak in the underlying commissural subnucleus of the NTS that contains specialized, fenestrated capillaries permeable to circulating hormones (Gross et al., 1990; Gross et al., 1991; Shaver et al., 1991). The significant rise in the number of Fos positive cells in the AP induced by ip secretin in both sham and vagotomized rats provides evidence for entrance of blood borne signal, which is consistent with the leaky BBB at this circumventricular organ. Secretin may act on its receptors after accessing to brain parenchyma as established for other gut peptides such as CCK that directly activates the AP neurons (Zajac et al., 1996). In the NTS, Fos positive cells induced by ip secretin were mainly distributed in the medial subnuclei and only few positive cells were located in the commissural subnucleus, suggesting that additional non-humoral pathways are involved.

The present study provides convergent evidence for a primary role of the vagus in mediating ip secretin-induced Fos expression in the NTS. First, there is anatomical support for the presence of secretin receptors in vagal afferents. By real-time-PCR and RT-PCR, we detected expression of secretin receptor in the nodose ganglia and found significantly higher levels of secretin receptor mRNA in theright compared to the left nodose ganglion. These results were reproducible when two different primers were used in the real-time-PCR and RT-PCR

respectively. Such a difference in the contents of the secretin receptor mRNA within the two ganglia may be attributed to the presence of 20% higher number of vagal sensory neurons in the right compared to the left nodose ganglion in rats (Carobi, 1996), or simply reflects higher mRNA concentrations in the right than left nodose ganglion. In other studies, secretin receptors have been visualized on vagal fibers by autoradiography after vagal ligation or axonal blockade, indicating that there is an axonal transport of secretin receptors and the accumulation of the receptors in vagal fibers innervating the forestomach (Kwon et al., 1999; Wang LM et al., 1995). Secondly, the distribution of Fos expression in the medial NTS induced by ip secretin is consistent with the major distribution of abdominal vagal afferent nerve terminals within this subnucleus (Rinaman et al., 1989). Secretin-induced Fos expression was mainly localized at the medial NTS areas receiving afferents of the vagus innervating the gut (Barraco et al., 1992). Lastly, subdiaphragmatic vagotomy blocked ip secretin-induced Fos expression in the NTS. Previous functional studies showed that subdiaphragmatic vagotomy completely blocked peripheral secretin induced inhibition of gastric acid secretion and motility (Li et al., 1998; Lu et al., 1995).

Our data also indicate that the vagal component recruited by ip secretin is not capsaicin sensitive. Pretreatment with capsaicin, which selectively destroys unmyelinated sensory fibers (Holzer, 1991), failed to influence the pattern and magnitude of Fos expression in the brain induced by secretin injected ip at 40 μg/kg. Likewise, other reports have shown that gastric inhibitory responses to secretin injected peripherally at supra-physiologic doses were not blocked by capsaicin pretreatment in rats (Kwon et al., 1999). By contrast, secretin injected iv at physiological or low doses acts through vagal capsaicin sensitive pathways to inhibit gastric motility and acid secretion as well as to stimulate pancreatic bicarbonate secretion and blood

flow (Kwon et al., 1999; Li et al., 1995; Li et al., 1998). Secretin iv infusion at 0.1 μg/kg/h reproduces physiological levels achieved after a lipid meal (Lu et al., 1995). Therefore doses used in the present study are supra-physiological and previously found to induce a peak blood levels similar to that observed during secretin treatments in humans (Goulet et al., 2003; Kaminska et al., 2002). In addition, the demonstration that truncal vagotomy reduced secretin receptor levels in the forestomach while perivagal capsaicin had no effect (Kwon et al., 1999) may have a bearing with the different influence of subdiaphragmatic vagotomy and capsaicin on ip secretin-induced Fos expression in specific brain nuclei. Neuroanatomical studies also established that most of the vagal afferent fibers derived from the nodose ganglia terminating in the upper gut are capsaicin resistant (Berthoud et al., 1997). Berthoud et al. (1997) reported that capsaicin pretreatment induces the disappearance of primary vagal afferent endings in the small and large intestine, while there was a 70-90% survival of esophageal and gastric vagal afferents forming intraganglionic laminar endings in the myenteric plexus and intramuscular endings or arrays in capsaicin-pretreated rats. Functional data also showed that the integrity of gastric tension-sensitive vagal afferents are maintained after capsaicin treatment, since gastric distention induces similar numbers of Fos positive neurons in the medial and central subnuclei of the NTS in rats with and without capsaicin pretreatment (Berthoud et al., 1997). Collectively these data indicate that ip secretin given at supra-physiological doses may interact with secretin receptors located on capsaicin resistant vagal afferents in the upper gut and thereby pharmacologically increase vagal afferent signaling inputs to the NTS.

The present demonstration that vagotomy prevents or reduces Fos induction not only in the NTS but also in other pontine and limbic nuclei, implies that the NTS conveys vagal afferent inputs to other nuclei through direct or indirect neural pathways. NTS neurons relay signals

received from the vagal afferents to higher brain structures via ascending adrenergic and noradrenergic pathways (Cunningham et al., 1990). Double labeling showed that ip secretin activates a large population of TH positive neurons in the NTS. Neurons in pontine PBel and LC, which are activated by ip secretin, are recipients of direct projections from the visceral sensory region of the NTS (Herbert et al., 1990). Anterograde tracing studies demonstrated that the NTS, particularly its dorsomedial and commissural subnuclei, send monosynaptic projections to the PBel (Herbert et al., 1990), and to dendrites of LC neurons (Van et al., 1999). The compact Fos expression selectively in the PBel after ip secretin may result from neuronal excitation by signals sent through convergent projections from the AP, NTS as well as the CeA that are involved in modulating the central response to viscerosensory inputs (Strain et al., 1990)(Asan, 1998; Krukoff et al., 1993; Riche et al., 1990). Likewise, the robust activation induced in the lateral part of the CeA may be related to the established strong projections from the PBel to the lateral zone of the CeA (Bernard et al., 1993; Krukoff et al., 1993), as well as from direct synaptic inputs from activated catecholaminergic neurons in the NTS and LC (Asan, 1998; Riche et al., 1990). In particular, retrograde tracing studies showed that TH positive neurons within the A2 group, rather than adrenergic neurons of the C2 group, provide the bulk of catecholaminergic input from the NTS to the CeA (Zardetto-Smith et al., 1990). Taken together these data suggest that the increased Fos expression in these specific pontine (LC, PBel) and limbic (CeA) neurons induced by ip secretin may result from incoming signals primarily from the NTS along with reciprocal projections among these nuclei (Curtis et al., 2002; Krukoff et al., 1993; Saha et al., 2000; Zardetto-Smith et al., 1990). The observation that Fos expression in these nuclei except the AP was abolished or strongly diminished by vagotomy supports this view. Moreover, it can be further argued that the significant activation of the AP but not the NTS by secretin after

vagotomy suggests that NTS activation by secretin is not mediated through the known AP projections to the NTS (Cunningham et al., 1994; Miceli et al., 1987; Shapiro et al., 1985; van der Kooy et al., 1983).

The high level of interconnectivity between the PBel, LC and CeA has been ascribed to augment the integrative processing of visceral sensory inputs and the relay of information in several autonomic regulatory mechanisms that elicit visceral reflex (Curtis et al., 2002). The vagal dependent activation of the catecholaminergic NTS neurons and CeA neurons demonstrated in the present study may modulate acquisition and expression of both autonomic visceral and behavioral learned emotional responses to threatening stimuli (LeDoux et al., 1988). It has been established that activation of noradrenergic neurons in the NTS enhances retention performance in emotionally arousing and spatial memory tasks (Clayton et al., 2000). Recent studies also showed that ip secretin dose-dependently decreased the magnitude of fear-potentiated startle in rats (Myers et al., 2003).

In summary, the present findings indicate that the major component of the signaling pathways whereby secretin injected ip at supra-physiological doses induced brain neuronal activation involves capsaicin-resistant vagal afferents. This is supported by the demonstration that the Fos expression in the NTS, DMV, LC, Bar, PBel and CeA induced by secretin injected ip at 40 μg/kg was completely or largely prevented by subdiaphragmatic vagotomy while capsaicin pretreatment had no effect. In addition, secretin receptor mRNA is expressed in the nodose ganglia that contain the perikaria of abdominal vagal afferents, indicating that secretin receptors on vagal afferent fibers may serve as target sites for ip secretin-induced signaling to the NTS. We showed also that activated neurons in the NTS include a large population of catecholaminergic neurons. Established direct or indirect projections between the NTS and

DMV, PBel, LC, CeA as well as interconnections among these nuclei may account for the vagal dependent Fos induction at these sites. We also found that ip secretin induces a largely non-vagal dependent Fos expression selectively in the AP, an area outside of the BBB, indicative of a direct access through blood born signaling. These observations may have implications in the understanding of the vagal dependent action of peripheral secretin on gastric and pancreatic functions and the underlying mechanisms through which peripheral secretin exerts central actions influencing behavior.

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Figure legends

Fig. 1. Dose-related increase of Fos expression in brain nuclei regulating autonomic function and behavior induced by ip secretin. Each column represents mean ± SEM of number of rats indicated in the bottom or above each column. * P< 0.05 compared with rats received vehicle ip injection.

Fig. 2. Photomicrographs of the medullary dorsal vagal complex (DVC) showing Fos immunoreactivity (dark blue in cell nucleus) in TH-containing neurons (cell plasma stained brown) in the NTS of rats received ip secretin (40 μg/kg) injection. Scale bar: 200 μm.

Fig. 3. Subdiaphragmatic vagotomy (-7 days) prevented the Fos induction by ip secretin in the observed brain nuclei (40 μg/kg). Each column represents mean ± SEM of number of rats indicated in the bottom or above each column. * P< 0.05 compared with sham-operated rats received vehicle ip injection; # P< 0.05 compared with sham-operated rats received secretin ip injection; @ P< 0.05 compared with vagotomized rats received vehicle ip injection.

Fig. 4. Photomicrographs of the CeA (top photos), and medullary NTS and DMV (bottom photos) showing the effect of subdiaphragmatic vagotomy on Fos expression at these areas induced by ip secretin (40 μg/kg). Scale bar: 200 μm.

Fig. 5. Secretin receptor gene expression in the nodose ganglia. The cerebellum and nodose ganglia were dissected from 6 rats (1-6). L: Left nodose ganglion. R: Right nodose ganglion. The control genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and acetic ribosomal protein (ARP) used in respective experiments were shown below the secretin receptor signals. 5A. RT-PCR analysis of secretin receptor mRNA using the secretin receptor sequence between TM-3 and TM-6 (amplicon size= 344 bp, right Panel). 5B. Real-time-PCR analysis of secretin

receptor mRNA. The amount of cerebellum total RNA used in the reverse transcription is as indicated. The third lane represents a negative control showing the lack of PCR products obtained when using non-reverse transcribed nodose ganglia RNA as a PCR template. The experiments were performed to amply the entire N-terminal extracellular domain (amplicon size= 492 bp, Left Panel). 5C. Densitometic measurement of relative secretin receptor mRNA levels in the left and right nodose ganglion from the RT-PCR gel images. All secR amplicons were sequenced to confirm analytical specificity. For each sample, data was normalized to control gene and this value adjusted so that left nodose ganglion had a mean relative mRNA level of 1. 5D. Real-time-PCR analysis of relative secretin receptor mRNA levels in the left and right nodose ganglia. Data are presented as the mean ± SEM.